

**Pulmonary fibrosis inducer, bleomycin, activates phospholipase D and generates bioactive lipid signal mediator, phosphatidic acid in lung microvascular endothelial cells**

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## Table of Contents

<b>Abstract.....</b>	<b>Page 4</b>
<b>Introduction.....</b>	<b>Page 6</b>
<b>Abbreviations.....</b>	<b>Page 5</b>
<b>Materials and Methods.....</b>	<b>Page 10</b>
<b>Results.....</b>	<b>Page 15</b>
<b>Discussion.....</b>	<b>Page 22</b>
<b>References.....</b>	<b>Page 27</b>
<b>Figure Legends.....</b>	<b>Page 35</b>
<b>Figures.....</b>	<b>Page 41</b>

## Abstract

The mechanisms of lung microvascular complications and pulmonary hypertension known to be associated with idiopathic pulmonary fibrosis (IPF), a debilitating lung disease, are not known. The bioactive lipid signal mediator, phosphatidic acid (PA), generated by phospholipase D (PLD), is emerging as a key player in vascular endothelial dysfunctions. Therefore, we investigated whether bleomycin, the widely used experimental IPF inducer, would be capable of activating PLD and generating PA in our established bovine lung microvascular EC (BLMVEC) model. Our results revealed that bleomycin induced the activation of PLD and generation of PA in a dose-dependent (0-100  $\mu$ g) and time-dependent (2-12 h) fashion that were significantly attenuated by the PLD-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI). PLD activation and PA generation induced by bleomycin (5  $\mu$ g) were significantly attenuated by the thiol protectant (N-acetylcysteine), antioxidants, and iron chelators, suggesting the role of reactive oxygen species (ROS), lipid peroxidation, and iron therein. Furthermore, our study demonstrated the formation of ROS and loss of glutathione (GSH) in cells following bleomycin treatment, confirming oxidative stress as a key player in the bleomycin-induced PLD activation and PA generation in ECs. More noticeably, PLD activation and PA generation were observed to occur upstream of cytotoxic response (cell morphology alterations or lactate dehydrogenase release) in BLMVECs, which was protected by FIPI. Overall, this study revealed novel information that bleomycin induced the redox-sensitive activation of PLD leading to the generation of PA, capable of inducing lung EC cytotoxicity, thus suggesting possible mechanism(s) of microvascular disorders encountered in IPF.

## **Abbreviations**

4-HNE – 4-Hydroxynonenal

BCA – Bicinchoninic Acid

BLMVECs – Bovine Lung Micro-Vascular Endothelial Cells

BSA – Bovine Serum Albumin

EC – Endothelial Cells

FIPI – 5-fluoro-2-indolyl des-chlorohalopemide

LDH – Lactate dehydrogenase

PLD – Phospholipase D

ROS – Reactive Oxygen Species

S.D. – Standard deviation

TBST – Tris-Buffered saline Tween-20

## Introduction

Cellular membranes, including the plasma membrane and membranes of organelles, consist of phospholipids, proteins, and carbohydrate moieties. The membrane phospholipids are critical for cell structure and function and the cellular homeostasis and survival. Cellular signaling is one of the crucial cell functions required for homeostasis. Enzymes belonging to the family of membrane phospholipid hydrolases called “phospholipases” are both the house-keeping enzymes as well as the cellular signal transduction-mediating enzymes. Phospholipases  $A_1$  (PLA<sub>1</sub>),  $A_2$  (PLA<sub>2</sub>), C (PLC), and D (PLD) act on the membrane phospholipids at specific sites to generate bioactive lipid signal mediators which induce cellular signals at targets leading to cellular functional responses (Steinhour et al., 2007). Phospholipase D (PLD) is one such membrane phospholipid hydrolase which generates a potent cellular bioactive lipid signal mediator, phosphatidic acid (PA), which, in turn is further metabolized into potent bioactive lipid signal mediators such as diacylglycerol (DAG) and lysophosphatidic acid (LPA) (Parinandi et al., 1999; Cummings et al., 2002). PLD has been shown to be involved in several pathophysiological conditions such as cardiovascular diseases and cancer (Tappia et al., 2006; Su et al., 2009). Thus, the PLD-generated bioactive lipid mediators (PA, DAG, and LPA) regulate the cellular cytoskeleton, especially the actin cytoskeleton, which leads to the regulation and/or alteration of the cell shape and movement.

PLD activation in mammalian cells is under the control of several regulators including hormones, growth factors, G protein-coupled receptors, and other bioactive signal mediators through the regulation of protein kinases such as protein kinase C, tyrosine kinase, and mitogen-activated protein kinases (Steinhour et al., 2007). Oxidative

stress and oxidant exposure (reactive oxygen species, ROS) are known to activate PLD in mammalian cells (Varadharaj et al., 2006). Vascular endothelial cells (ECs), the inner monolayer lining of blood vessels which form the barrier, are susceptible to oxidative stress that leads to vascular endothelial cell disruption and vascular leak. Oxidative stress-induced activation of PLD and associated lipid-signaling enzymes are emerging as responsible players for EC dysfunction leading to pathophysiological conditions in lung vasculature during oxidant exposure such as infection, pulmonary hypertension, lung fibrosis, and hyperoxic lung damage. Oxidative stress also leads to the redox perturbation in the cells such as alterations in the soluble thiols (glutathione, GSH) and protein thiols (Parinandi et al., 1999). However, the thiol redox-modulation of oxidative stress-mediated activation of PLD in lung ECs is not completely understood. Therefore, here, it is hypothesized that oxidative stress induced by oxidants induces an imbalance in the thiol redox of the lung vascular ECs so that the redox-sensitive PLD is activated, resulting in the generation of the bioactive lipid mediator, PA, that ultimately signals the vascular EC barrier dysfunction.

Idiopathic pulmonary fibrosis (IPF) is a degenerative, chronic, and progressive fibrosing lung disorder of the tissue that lines and separates the alveoli with unknown etiology (Kinnula et al., 2005). The prevalence of IPF in the US is estimated in the range of 35,000 to 55,000 cases as recorded in 2005 (Zisman et al., 2005). Extended exposure to environmental and occupational agents including metal dust, wood dust, and stone dust have been associated with the tissue damage that leads to IPF among the idiopathic lung diseases (Taskar and Coultas, 2008; Wilson and Wynn, 2009). IPF is a progressive interstitial lung disorder where the tissue that lines and separates the alveoli becomes

scarred due to lung damage. This scarring causes the tissue to become inelastic and hard. The buildup of scar tissue causes difficulties in breathing and results in respiratory failure. Interstitial lung diseases (ILD) including the sarcoidosis, IPF, and pulmonary Langerhans cell histiocytosis have been shown to be associated with pulmonary hypertension (PH) (Ryu et al., 2007; Cordier, 2008). Lung parenchymal and vascular remodeling indicates the high prevalence (30-40%) of PH among the ILD patients (Behr and Ryu, 2008). Although both the lung epithelium and endothelium have been shown to be the critical cellular players in IPF, microvascular injury has been shown as an initial event in lungs during IPF (Calabrese et al., 2005). In addition, microvascular injury has been emphasized as an important event in the evolution of IPF (Magro et al., 2003). Therefore, the lung microvasculature, specifically the lung microvascular endothelium, is apparently a target in the lung fibrotic events. Bleomycin is widely used as an experimental lung fibrosis inducer (Tager et al., 2008). Since vascular endothelial alterations are encountered in lung fibrosis, both experimental and disease settings, we rationalized that bleomycin is a suitable model lung fibrosis inducer to study the vascular endothelial alterations through PLD signaling in cultured lung ECs. This will provide an understanding of PLD signaling-mediated lung EC damage associated with IPF.

Several cellular signaling mechanisms involving caveolae, caveolins, protein kinases, focal adhesion kinase, protein kinase B, and mitogen-activated protein kinases (MAPKs) have been shown to operate in IPF (Gosens et al., 2008; Garneau-Tsodikova and Thannickal, 2008; Chopra et al., 2008). Studies with experimental models have revealed the roles of oxidative stress and antioxidant imbalance in the initiation and progression of IPF. Accordingly, redox modulatory therapy has been proposed for the



treatment of the disease (Kinnula et al., 2005; Kinnula and Myllarniemi, 2008). As the lung microvasculature and vascular endothelium are critical regions in IPF, oxidative stress and redox alterations appear to drive the initiation and progression of IPF, and oxidants are known to activate the lung vascular endothelial PLD leading to the generation of bioactive lipid mediators, it is compelling to rationalize that oxidant-mediated lung microvascular endothelial PLD signaling is likely to play a crucial role in the initiation and propagation of IPF. To date, no reports have been made on the activation of PLD by the experimental lung fibrosis inducer, bleomycin, in lung cells including the vascular endothelium. Therefore, here, we have hypothesized that bleomycin induces oxidant-mediated and redox-dependent PLD activation upstream that leads to the cytotoxicity downstream through the PLD-generated bioactive lipid mediator, PA in the lung microvascular endothelial cells (ECs) in culture. To test this hypothesis, we used the well-established bovine lung microvascular ECs (BLMVECs) and exposed them to the experimental lung fibrosis inducer, bleomycin, to establish the thiol-redox regulation of PLD activation in lung ECs and define the connection between the upstream PLD activation and downstream cytotoxicity that could be responsible for the oxidant-mediated lung vascular dysfunction similar to that encountered in IPF conditions. For the first time, the results of the current study revealed that the experimental lung fibrosis inducer, bleomycin, induced the upstream activation of PLD and generated the bioactive lipid signal mediator, PA, in oxidant-mediated and redox-dependent mechanism that caused cytotoxicity in the lung microvascular ECs in culture, suggesting the possible role of PLD in IPF.

## **Materials and Methods**

### *Materials*

BLMVECs (passage 4) were purchased from VEC Technologies (NY, USA). Phosphate-buffered saline (PBS) was obtained from Biofluids Inc. (Rockville, MD). Minimal essential medium (MEM), nonessential amino acids, trypsin, fetal bovine serum (FBS), penicillin/streptomycin, DMEM phosphate-free modified medium, tissue culture reagents, and analytical reagents of highest purity were all purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylbutanol (Pbt) was obtained from Avanti Polar Lipids (Alabaster, AL). [ $^{32}$ P] Orthophosphate (carrier-free) was obtained from New England Nuclear (Wilmington, DE). Bleomycin was obtained from Teva Parenteral Medicines (Irvine, CA). Desferal was obtained from Calbiochem (San Diego, CA). Polyclonal antibody to 4-Hydroxynonenal (anti-HNE) was obtained from Enzo Life Sciences (Farmingdale, NY). Endothelial cell growth factor was obtained from Upstate Biotechnology (Lake Place, NY). 5-Fluoro-2-indolyl des-chlorhalopemide hydrochloride hydrate (FIPI) was obtained from Dr. Andrew J. Morris of University of Kentucky. GSH-Glo Glutathione Assay kit was obtained from Promega Corporation (Madison, WI)

### *Cell culture*

BLMVECs were grown to confluence in MEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and streptomycin, 5  $\mu$ g/ml endothelial cell growth factor and 1% (v/v) nonessential amino acids at 37 °C under a humidified 95% air-5% CO<sub>2</sub> atmosphere as described earlier (Steinhour et al., 2007). BLMVECs, from passages 7 to 15, were used in the experiments. ECs cultured in 35-mm or 60-mm sterile dishes or T-75 cm sterile flasks to ~95% confluence under a humidified 95% air-5% CO<sub>2</sub>

atmosphere were used for treatments with bleomycin and desired pharmacological agents. MEM containing bleomycin and other pharmacological agents were carefully adjusted to pH 7.4 for cellular treatments.

#### *Phospholipase D activation in intact ECs*

ECs in 35-mm dishes ( $5 \times 10^5$  cells/dish) were labeled with [ $^{32}\text{P}$ ]orthophosphate (5  $\mu\text{Ci/ml}$ ) in DMEM phosphate-free medium containing 2% (v/v) fetal bovine serum for 12-14 h (Varadharaj et al., 2006). Cells were washed with MEM and incubated at 37 °C in 1 ml of MEM containing 0.05% (v/v) 1-butanol in absence and presence of desired concentrations of bleomycin for different lengths of time under a humidified 95% air-5%  $\text{CO}_2$  atmosphere. In some experiments, wherever required, ECs were pretreated for 2 h or 12 h with selected pharmacological inhibitors prior to exposure to bleomycin or co-treated with bleomycin and pharmacological inhibitors for the desired lengths of incubation time. The incubations were terminated by addition of 1 mL methanol:HCl (100:1, by vol.). Lipids were extracted essentially according to the method of Bligh and Dyer procedure as described previously (Steinhour et al., 2007). [ $^{32}\text{P}$ ]-Labeled phosphatidylbutanol (PBt) formed as a result of PLD activation and transphosphatidyl transfer reaction, as an index of PLD activity in intact cells, was separated by thin-layer chromatography (TLC) (Varadharaj et al., 2006; Steinhour et al., 2007). Radioactivity associated with the [ $^{32}\text{P}$ ]-PBt was quantified by liquid scintillation counting and data were expressed as DPM normalized to  $10^6$  counts in the total cellular lipid extract or as % of control (vehicle-treated cells).

### *Reactive oxygen species (ROS) measurement by DCFDA fluorescence*

Formation of ROS in BLMVECs in 35-mm dishes ( $5 \times 10^5$  cells/dish) was determined by DCFDA fluorescence in cells preloaded with 10  $\mu$ M DCFDA for 30 min in complete MEM at 37° C in a 95% air-5% CO<sub>2</sub> environment prior to exposure to bleomycin for 1 h. At the end of exposure to bleomycin, the dishes containing the cells were either photographed using Olympus 1x50 fluorescent microscope with excitation and emission set at 490 nm and 530 nm respectively, or the cells were detached with a Teflon cell scraper. The medium which contained the cells was transferred to 1.5 mL microcentrifuge tubes and centrifuged at 8000 x g for 10 min at 4° C. The supernatant was aspirated and the cell pellet was washed twice with ice-cold phosphate-buffered saline (PBS). To prepare cell lysates, pellets were sonicated on ice with a probe sonicator at a setting of 2 for 15 s in 500  $\mu$ L of ice-cold PBS. Fluorescence of oxidized DCFDA in cell lysates, an index of formation of ROS, was measured on a Bio-Tex ELx808 fluorescent plate reader set at 490 nm and 530 nm respectively, using appropriate blanks. The extent of ROS formation was expressed as the arbitrary fluorescence units.

(Hagele et al., 2007)

### *GSH determination*

Intracellular soluble thiol levels were determined using the GSH-Glo Glutathione Assay. BLMVECs grown up to 90% confluence in 96 well plates were treated with MEM containing desired concentrations of bleomycin for 12 h under a humidified 95% air-5% CO<sub>2</sub> atmosphere. Following incubation, intracellular GSH levels were determined according to the manufacturer's recommendations (Madison, WI).

### *Immunofluorescence microscopy*

BLMVECs cultured on sterile coverslips (Harvard Apparatus, 22 mm<sup>2</sup>) in 35-mm sterile dishes at a density of 10<sup>4</sup> cells/dish were treated with MEM alone and MEM containing bleomycin for 12 h under a humidified atmosphere of 95% air-5% CO<sub>2</sub> at 37 °C. At the end of the incubation period, cells attached to coverslips were washed with 1x PBS and fixed with 3.7% of para-formaldehyde for 10 min, permeabilized with 0.25% Triton X-100 in TBST containing 0.01% Tween-20 for 5 min, and blocked for 30 min with 1% BSA in 0.01% TBST and incubated for 12 h at room temperature with rabbit primary anti-4-HNE at a dilution of 1:200 for the visualization of 4-HNE formation. Following treatment of cells with the chosen primary antibodies, they were incubated with secondary anti-rabbit AlexaFluor 488-conjugated antibodies (1:100 dilution), for 1 h at room temperature. The coverslips with cells were then mounted on a glass slide with the antifade mounting medium, Fluoromount-G, viewed with Ziess Confocal microscope at a magnification of 60 X, and pictures were captured digitally.

### *Morphology assay of cytotoxicity*

Morphological changes in BLMVECs grown in 35-mm dishes up to 70% confluence, following their exposure to bleomycin (5 µg, 10 µg, and 100 µg) for specific times, were examined as an index of cytotoxicity. Images of cell morphology were digitally captured with the Olympus 1x50 at 20x magnification.

### *Lactate dehydrogenase release (LDH) assay of cytotoxicity*

Cytotoxicity in BLMVECs was determined by assaying the release of LDH from cells according to our previously published method (Mazerik et al. 2007). BLMVECs grown up to 90% confluence in 35-mm dishes were treated with MEM containing desired

concentrations of bleomycin for different lengths of time under a humidified 95% air-5% CO<sub>2</sub> atmosphere. In some experiments, wherever required, ECs were pretreated for 12 h with selected pharmacological inhibitors prior to exposure to bleomycin or co-treated with bleomycin and pharmacological inhibitors for 12 h. At the end of treatment, the medium was collected and LDH released into the medium was determined spectrophotometrically according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO). The experiments were terminated with the addition of 1 N HCl.

#### *Protein determination*

Protein was determined by BCA protein assay (Pierce).

#### *Statistical analysis*

All experiments were done in triplicate. Data were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out by ANOVA using SigmaStat (Jandel). The level of statistical significance was taken as  $P < 0.05$ .

## Results

### *Bleomycin induces PLD activation in a dose- and time-dependent fashion in ECs*

Bleomycin, in a dose-dependent manner (0-100  $\mu\text{g}$ ), at 4 and 12 h of incubation, induced significant activation of PLD in BLMVECs as compared with the vehicle-treated control cells (2-, 2-, and 3-fold for 4 h and 5-, 6-, and 12-fold for 12 h at 5, 10, and 100  $\mu\text{g}$ , respectively) (Fig. 1A). Incubation of ECs with bleomycin (5  $\mu\text{g}$ ) for 4, 8, 12, and 24 h induced significant increases (2-, 3-, 4-, and 3-fold) in the activation of PLD as compared with that in the cells exposed to the MEM alone (Fig. 1B).

### *Thiol-protectants attenuate bleomycin-induced PLD activation in ECs*

Altered thiol redox has been shown to activate PLD in ECs. Therefore, to establish the role of thiols in bleomycin-induced PLD activation in ECs, here, the effects of well-established thiol protective agents (NAC and DTT) were investigated on PLD activation induced by bleomycin. Cells were pretreated for 2 h with MEM alone or MEM containing the chosen thiol-protectant (0.5, 1 and 5 mM) and then co-treated for 12 h with bleomycin (5  $\mu\text{g}$ ) and the chosen thiol-protectant. NAC, a widely used thiol protectant and antioxidant, caused effective and significant attenuation of bleomycin-induced PLD activation in ECs (22, 70, and 97% of inhibition for concentrations of 0.5, 1, and 5 mM respectively) (Fig. 2A). DTT, a sulfhydryl protective agent, also offered effective and significant attenuation of bleomycin-induced PLD activation in ECs (43, 67, and 65% of inhibition for concentrations of 0.5, 1, and 5 mM respectively) (Fig. 2B). These results revealed that thiol-protectants effectively attenuated bleomycin-induced

PLD activation in ECs, further suggesting the involvement of cellular thiol status in the bleomycin-induced activation of PLD in ECs.

*Heavy metal chelators attenuate bleomycin-induced PLD activation in ECs.*

Oxidative stress and ROS production has been shown to activate PLD in ECs. Heavy metals, particularly  $\text{Fe}^{2+}$ , have been involved in the generation of ROS especially through fenton-type reactions. Bleomycin complexes with iron to become an active drug. Therefore, to establish the role of oxidative stress in bleomycin-induced PLD activation in ECs, here, the effects of well-established heavy metal chelators (EDTA, Desferal, and DMSA) were investigated on PLD activation induced by bleomycin. Cells were pretreated for 2 h with MEM alone or MEM containing the chosen heavy metal chelator (2 mM for EDTA and Desferal, and 0.5, 1, 5 mM for DMSA) and then co-treated for 12 h with bleomycin (5  $\mu\text{g}$ ) and the chosen metal chelator. EDTA and Desferal, widely used  $\text{Fe}^{2+}$  chelators, caused effective and significant attenuation of bleomycin-induced PLD activation in ECs (32 and 27% of inhibition respectively) (Fig. 3A and 3B). DMSA, a heavy metal chelator and a thiol-redox stabilizer, also offered effective and significant attenuation of bleomycin-induced PLD activation in ECs (39, 64, and 64% of inhibition for concentrations of 0.5, 1, and 5 mM respectively) (Fig. 3C). These results revealed that heavy metal chelators, especially  $\text{Fe}^{2+}$ -specific chelators, effectively attenuated bleomycin-induced PLD activation in ECs, further suggesting the involvement of ROS production in the bleomycin-induced activation of PLD in ECs.



*Antioxidants attenuate bleomycin-induced PLD activation in ECs.*

Oxidative stress, ROS production, and altered thiol redox has been shown to activate PLD in ECs. Moreover, bleomycin is established as an oxidant drug. We also found, previously in this study, that heavy metal chelators and thiol protectants effectively and significantly attenuated bleomycin-induced PLD activation in ECs. Therefore, in order to establish the role of oxidative stress in bleomycin-induced PLD activation in ECs, here, the effects of widely accepted antioxidants (PDTC, Propyl Gallate, Vitamin C, and MnTBAP) were investigated on PLD activation induced by bleomycin. Cells were pretreated for 2 h with MEM alone or MEM containing the chosen antioxidant (0.5, 1, and 5 mM for PDTC, 500  $\mu$ M for Propyl Gallate and Vitamin C, and 20  $\mu$ M for MnTBAP) and then co-treated for 12 h with bleomycin (5  $\mu$ g) and the chosen antioxidant. PDTC caused effective and significant attenuation of bleomycin-induced PLD activation in ECs (25, 49, and 72% of inhibition at 0.5, 1, and 5 mM respectively) (Fig. 4A). Propyl Gallate, a phenolic antioxidant, and Vitamin C also offered effective and significant attenuation of bleomycin-induced PLD activation in ECs (49, and 46% respectively) (Fig. 4B and 4C). MnTBAP, a superoxide scavenger, also offered effective and significant attenuation of bleomycin-induced PLD activation in ECs (21%) (Fig. 4D). These results revealed that antioxidants effectively attenuated bleomycin-induced PLD activation in ECs, further suggesting the involvement of ROS in the bleomycin-induced activation of PLD in ECs.

*Lipid peroxidation inhibitors attenuate bleomycin-induced PLD activation in ECs.*

Oxidative stress and ROS production has been shown to induce lipid peroxidation in ECs. Therefore, in order to establish the role of lipid peroxidation in bleomycin-induced PLD activation in ECs, here, the effects of lipid peroxidation antioxidants (NDGA and Trolox) were investigated on PLD activation induced by bleomycin. Cells were pretreated for 2 h with MEM alone or MEM containing the chosen lipid peroxidation antioxidant (10, 20, and 50  $\mu$ M for NDGA, and 100, 200, and 500  $\mu$ M for Trolox) and then co-treated for 12 h with bleomycin (5  $\mu$ g) and the chosen lipid peroxidation antioxidant. NDGA, a plant derived polyphenol, caused effective and significant attenuation of bleomycin-induced PLD activation in ECs (45, 72 and 67% of inhibition for 10, 20, and 50  $\mu$ M NDGA, respectively) (Fig. 5A). Trolox, a water soluble form of vitamin E, also offered effective and significant attenuation of bleomycin-induced PLD activation in ECs (61, 69, and 71% of inhibition for concentrations of 100, 200, and 500  $\mu$ M Trolox, respectively) (Fig. 5B). These results revealed that lipid peroxidation antioxidants effectively attenuated bleomycin-induced PLD activation in ECs, further suggesting the involvement of lipid peroxidation in the bleomycin-induced activation of PLD in ECs.

*Bleomycin induces ROS generation in ECs.*

Bleomycin's activity as a drug has been attributed to its ROS-forming nature. Earlier in the study we showed that bleomycin-induced PLD activation was attenuated by antioxidants, thus it was hypothesized that bleomycin would induce ROS formation in ECs. DCFDA is a lipid-soluble probe that becomes fluorescent when oxidized by

multiple forms of ROS. DCFDA fluorescence was measured as an index of ROS generation in ECs. Bleomycin, in a dose-dependent manner (0-100  $\mu\text{g}$ ), at 1 h of incubation, induced significant ROS formation in BLMVECs as compared with the vehicle-treated control cells as seen by the relative fluorescence. Bleomycin, at 0.5 h of incubation, effectively induced ROS formation in BLMVECs as compared with the vehicle-treated control cells (1.5-, 1.8-, and 2-fold at 5, 10, and 100  $\mu\text{g}$ , respectively).

*Bleomycin causes GSH depletion in ECs.*

Earlier in the study, we showed that thiol-protectants attenuated bleomycin-induced PLD activation in ECs. Therefore, we hypothesized that bleomycin depletes intracellular glutathione (GSH). To test this postulate, intracellular soluble thiols, GSH were measured. Bleomycin in a dose-dependent manner induced significant GSH depletion in BLMVECs after a 12 h incubation as compared with the vehicle-treated control cells (60, 57, and 45% of the control for the 5, 10, and 100  $\mu\text{g}$ , respectively).

*Bleomycin induces lipid peroxidation in ECs.*

Earlier in the study, we showed that bleomycin causes the production of ROS in ECs. ROS is known to cause lipid peroxidation of polyunsaturated fatty acids and produce highly reactive carbonyls such as 4-HNE. To observe if bleomycin induced lipid peroxidation in ECs, cells were treated with bleomycin for 12 h, fixed, and stained with anti-4-HNE antibodies. Using confocal microscopy, we showed that bleomycin induced the formation of 4HNE (as an index of lipid peroxidation) in a dose-dependant manner.

*PLD inhibitor, FIPI, protects against bleomycin-induced PLD activation in ECs.*

We speculated that the cytotoxic effects of bleomycin were a result of the PLD-generated PA. To test this premise, we wanted to inhibit PLD activity using a novel PLD inhibitor, FIPI. To validate the PLD inhibitor, FIPI (250, 500, and 1000 nM) was added to ECs for 12 h while they were being labeled with [ $^{32}$ P]-orthophosphate. The ECs were then co-treated with FIPI and bleomycin (5  $\mu$ g) for 12 h. The results revealed that FIPI significantly attenuated bleomycin-induced PLD activation (25, 42, and 68 % at 250, 500, and 1000 nM respectively). FIPI was also found to inhibit the basal normal PLD activity (27, 47, and 62% at 250, 500, and 1000 nM respectively).

*Bleomycin induces morphological alterations in ECs.*

Since PLD is a bioactive lipid-signaling enzyme that plays an important role in EC function, we investigated the effects of bleomycin on the cell morphology as an index of disruption of cellular structure and cytotoxicity. Cells were treated with bleomycin (5, 10, and 100  $\mu$ g) for 4, 8, 12, and 24 h before morphological images were taken digitally. Bleomycin in a time- and dose-dependant manner causes morphological alteration in ECs. The results indicate that even with 5  $\mu$ g of bleomycin, the morphological alterations begin before 4 h and the barrier is completely destroyed by 12 h.

*PLD inhibitor protects against bleomycin-induced morphological alterations in ECs.*

To investigate whether PLD activation has a role in the bleomycin-induced morphological alterations, we inhibited the PLD activity using FIPI and measured the bleomycin-induced morphological alterations. Cells were pretreated with FIPI (1  $\mu$ M)

for 12 h, and co-treated with bleomycin (5  $\mu$ g) for 4, 8, 12 and 24 h before morphological images were taken digitally. FIPI was found to significantly attenuate bleomycin-induced morphological alterations at all time-points, implicating PLD activation in the bleomycin-induced morphological alterations.

*Bleomycin induces cytotoxicity in ECs.*

As the PLD-generated PA was associated with cytotoxicity, another measure of cytotoxicity, the release of LDH was also measured. Bleomycin was found to increase the release of LDH in a dose- and time-dependant fashion. Bleomycin (5, 10, and 100  $\mu$ g) caused LDH release of 20, 21, and 103% respectively at 8 h, 234, 250, and 266% respectively at 12 h, and 220, 244, and 302 % respectively at 24 h. These results indicate that bleomycin induces significant LDH release in ECs by 12 h.

*PLD inhibitor, FIPI, protects against bleomycin-induced cytotoxicity in ECs.*

Because the PA generated from PC through PLD has been implicated in many physiological disorders, we investigated if that the cytotoxic effects of bleomycin were caused by the PLD-generated PA. To measure the cytotoxic effects of the PLD generated PA, we assayed the bleomycin-induced LDH release in the ECs after inhibiting the PLD using FIPI. ECs were pretreated with FIPI (250, 500, and 1000 nM) for 12 h, before being exposed to bleomycin (5  $\mu$ g) for 12 h. FIPI (250, 500, and 1000 nM) inhibited PLD-induced LDH release (23, 36, and 45%) implicating the role of PLD-generated PA in the bleomycin-induced cytotoxicity in ECs.

**Discussion:**

The results of the current study revealed that bleomycin at pharmacological doses ( $\mu$ U) induced the activation of PLD, which was attenuated by antioxidants, thiol-protectants, and iron chelators, suggesting the upstream roles of oxidants and altered redox signaling in the activation of PLD in BLMVECs. This was further confirmed by the enhanced generation of ROS and decrease in thiol levels upon treatment of BLMVECs with bleomycin. FIPI, a PLD-specific inhibitor, caused complete inhibition of the bleomycin-induced activation of PLD and also offered protection against the cytotoxic effects of bleomycin in BLMVECs. Taken together, these results demonstrated the role of PLD-generated PA in the bleomycin-induced lung microvascular EC damage.

Phospholipases are enzymes which specifically hydrolyze the membrane phospholipids and generate bioactive lipid second messengers, which may play a vital role in cell signaling (Devecha and Irvine, 1995). Phospholipase D (PLD) is one such lipid-signaling enzyme ubiquitously present in all mammalian cells that preferentially hydrolyzes phosphatidylcholine (PC) thus generating phosphatidic acid (PA) and choline (Exton, 1999). PA is further metabolized to either 1,2-diacylglycerol (DAG) by phosphatidate phosphohydrolase or lysophosphatidic acid (LPA) by phospholipase A<sub>1</sub>/A<sub>2</sub> (Natarajan, 1995; Brindley and Waggoner, 1996). Agonist-mediated activation of PLD plays a pivotal role in signal transduction in mammalian cells (Exton, 1997; Singer et al., 1996). Several functions, including the promotion of mitogenesis in fibroblasts, stimulation of oxidative stress in neutrophils, increase of intracellular calcium, activation of protein kinases and phospholipases have been attributed to the signaling actions of PA and LPA (Exton, 1999). Two major forms of PLD, hPLD<sub>1</sub> and hPLD<sub>2</sub>, have been cloned

in mammalian cells (Hammond et al., 1995; Colley et al., 1997; Lopez et al., 1998). Even though the two isoforms of PLD catalyze hydrolysis of PC to PA, they are selectively activated by various cofactors such as Arf, Rho, Cdc42, and detergents (Liscovitch and Chalifa-Caspi, 1996; Houle and Bourgoignie, 1999). Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), a lipid mediator, activates both hPLD<sub>1</sub> and hPLD<sub>2</sub> in cell-free preparations. The results of the current work showed that bleomycin activated PLD in the BLMVECs generating the lipid signal mediator, PA. This could be possible through the actions of both PLD<sub>1</sub> and PLD<sub>2</sub> isoforms as the presence of both the isoforms in ECs has been detected (Parinandi et al., 2001). However, the relative activity of these isoforms in the bleomycin-induced formation of PA is yet to be determined.

Earlier studies have shown that reactive oxygen species (ROS) such as hydrogen peroxide, fatty acid hydroperoxide, 4-hydroxynonenal, and oxidized-LDL stimulated PLD in vascular ECs, smooth muscle cells, and fibroblasts (Natarajan and Garcia, 1993a; Natarajan et al., 1998; Natarajan et al., 1993b; Natarajan et al., 1993c; Kiss and Anderson, 1994; Min et al., 1998). The ROS-mediated PLD activation is attenuated by tyrosine kinase inhibitors and pretreatment of ECs with protein tyrosine phosphatase inhibitors such as vanadate, phenylarsine oxide, or diamide increases ROS-induced PLD activation several-fold, suggesting a role for protein tyrosine phosphorylation in PLD activation (Natarajan et al., 1996a and 1996b; Natarajan et al. 1998). In addition to tyrosine kinase inhibitors, PLD activation by oxidants was also attenuated by antioxidants, indicating redox regulation of PLD (Parinandi et al., 1999). Recently, we reported that mercury-activated PLD in the vascular ECs through thiol-redox alteration and generation of ROS (Hagele et al., 2007). Antioxidants including the thiol protectants

have been shown to attenuate the pro-oxidant-induced activation of PLD in lung microvascular ECs (Steinhour et al., 2008) suggesting the role of ROS and thiol-redox in the enzyme activation. Along these lines, the results of the current study also revealed that the bleomycin-induced PLD activation was regulated by the ROS, thiol-redox perturbation, and lipid peroxidation in BLMVECs. The results of the current study also suggested oxidant-induced and thiol-regulated signaling in the bleomycin-induced activation of PLD and generation of PA in the lung microvascular ECs. However, the regulation of PLD activation by different stimuli is complex and involves changes in intracellular  $\text{Ca}^{2+}$ , protein kinase C (PKC), heterotrimeric G proteins, small molecular weight G proteins, and protein tyrosine kinases/protein tyrosine phosphatases (Parinandi et al., 2001; Varadharaj et al., 2006). The precise signaling pathway responsible for mediating the oxidant-induced and thiol-regulated activation of PLD caused by bleomycin in BLMVECs needs to be established.

Bleomycin is an oxidant drug and has been commonly used to induce pulmonary fibrosis in animal models (Moeller et al., 2008; Moore and Hogaboam, 2008). Bleomycin is well known for its action of inducing oxidative stress and redox alteration both *in vivo* and *in vitro* (Kinnula and Myllarniemi, 2008; Iyer et al., 2009). Oxidation of extracellular cysteine and alteration of redox state in lung, induction of apoptosis and senescence in lung cells, and disruption of iron homeostasis due to bleomycin exposure have been noticed in relation to the bleomycin-induced pulmonary fibrosis (Iyer et al., 2009; Kasper and Barth, 2009; Mungunsukh et al., 2010; Ghio, 2009). Formation and role of lipid-derived free radicals have been observed in bleomycin-induced lung injury indicating the role of lipid peroxidation in the pulmonary toxicity of bleomycin (Sato et al., 2008). Free



radical quenchers and lipid peroxidation chain-breaking antioxidants such as  $\alpha$ -tocopherol has also been reported to protect against the bleomycin-induced lung injury suggesting the role of lipid peroxidation and lipid radicals in the pulmonary toxicity of the drug (Suntres and Shek, 1997). Resveratrol, a phytochemical and antioxidant, has been shown to alleviate the bleomycin-induced lung injury in rats *in vivo* (Sener et al., 2007). The thiol protectant, N-acetylcysteine, and the iron chelator, deferoxamine, have been observed to attenuate the bleomycin-induced oxidative stress and lung injury (Teixeira et al., 2008). Thus, these reports support our current findings that bleomycin induces oxidative stress, alters thiol-redox status, activates PLD, and causes cytotoxicity in a redox-dependent pathway in BLMVECs and further provides evidence in favor of the protection of the bleomycin-induced adverse effects in the cells offered by antioxidants, thiol protectants, and iron chelators.

One of the key findings of the current study was the role of PLD-generated PA in the cytotoxicity of bleomycin in BLMVECs. This was further confirmed by the use of the novel PLD-specific inhibitor FIPI (Su et al., 2009), which not only completely inhibited the bleomycin-induced activation of PLD but also attenuated the cytotoxicity of bleomycin in the lung microvascular ECs. This observation further established a link between the upstream activation of PLD and downstream cytotoxicity exerted by bleomycin in BLMVECs, thus offering evidence in favor of the role of the PLD-generated bioactive lipid mediator PA therein. As the PLD-generated PA can further undergo enzymatic conversion into DAG and/or LPA, it was not clear whether the cytotoxicity-inducing bioactive lipid generated by PLD through bleomycin action was PA or DAG or LPA or combination of those. PLD-generated PA has been identified as the

precursor of LPA formation through the actions of PLA<sub>1</sub>/PLA<sub>2</sub> (Aoki, 2004; Aoki et al., 2008). LPA receptors, members of the G protein-coupled receptors, have been shown to be involved in several physiological and pathophysiological states (Aoki et al., 2008; Hama and Aoki, 2010). LPA receptors 1 and 2 have been identified to play roles in regulation of vascular injury (Panchatcharam et al., 2008). The role of LPA receptor 1 linking pulmonary fibrosis to lung injury towards causing fibroblast recruitment and vascular leak in the bleomycin model of pulmonary fibrosis mouse model has been unequivocally elucidated (Tager et al., 2008). Nevertheless, the sources of LPA in these studies have not been identified, at least in the bleomycin pulmonary fibrosis model. On the other hand, the current study indicated PLD as a possible source of PA that could be very well converted into LPA, another potent bioactive lipid mediator, which could be responsible for the observed bleomycin-induced vascular EC damage. Also, it should be emphasized that PLD-derived PA could have also exerted its cellular effects through PA-dependent or DAG-mediated signaling cascades.

Overall, the current study offered a novel signaling pathway of bleomycin-induced lung microvascular EC damage, involving the oxidative stress-mediated and thiol redox-regulated PLD activation and generation of the bioactive lipid mediator PA. Furthermore, the findings of the current study also identified PLD as a possible target for the therapeutic intervention of IPF.

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## Figure Legends

**Hypothesis:** It is hypothesized that the lung fibrosis inducer, bleomycin, will cause the formation of ROS, thiol-redox alterations and PLD activation to produce bioactive lipid signal mediator PA. PA will then cause further endothelial damage and barrier dysfunction.

**Figure 1:** Bleomycin induces PLD activation in a dose- and time-dependant fashion in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}\text{P}$ ]orthophosphate in DMEM phosphate-free medium for 12 h. Following [ $^{32}\text{P}$ ]orthophosphate labeling, cells were treated with different concentrations (0-100  $\mu\text{U}$ ) of bleomycin (**A**), or for different time periods (4-24 h; **B**) in MEM containing 0.05% 1-butanol. At the end of incubation period, [ $^{32}\text{P}$ ]PBt formed was determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells not treated with bleomycin.

**Figure 2:** Thiol-protectants attenuate bleomycin-induced PLD activation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}\text{P}$ ]orthophosphate in DMEM phosphate-free medium for 12 h. Following [ $^{32}\text{P}$ ]orthophosphate labeling, cells were pretreated for 2 h with MEM or MEM containing NAC (0.5, 1, and 5 mM; **A**) or DTT (0.5, 1, and 5 mM; **B**) and then subjected to co-treatment with vehicle or bleomycin (5  $\mu\text{U}$ ) for 12 h in presence of 0.05% (v/v) 1-Butanol. At the end of incubation period, [ $^{32}\text{P}$ ]PBt formed was determined as described under Materials and Methods. Data

represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Figure 3:** Heavy metal chelators attenuate bleomycin-induced PLD activation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}$ P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [ $^{32}$ P]orthophosphate labeling, cells were pretreated for 2 h with MEM or MEM containing EDTA (2 mM; **A**) or Desferal (2 mM; **B**) or DMSA (0.5, 1, 5 mM; **C**) and then subjected to co-treatment with vehicle or bleomycin (5  $\mu$ U) for 12 h in presence of 0.05% (v/v) 1-Butanol. At the end of incubation period, [ $^{32}$ P]PBt formed was determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Figure 4:** Antioxidants attenuate bleomycin-induced PLD activation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}$ P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [ $^{32}$ P]orthophosphate labeling, cells were pretreated for 2 h with MEM or MEM containing PDTC (0.5, 1, 5 mM; **A**) or Propyl Gallate (500  $\mu$ M; **B**) or Vitamin C (500  $\mu$ M; **C**) or MnTBAP (20  $\mu$ M; **D**) and then subjected to co-treatment with vehicle or bleomycin (5  $\mu$ U) for 12 h in presence of 0.05% (v/v) 1-Butanol. At the end of incubation period, [ $^{32}$ P]PBt formed was determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells

treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Figure 5:** Lipid peroxidation inhibitors attenuate bleomycin-induced PLD activation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}$ P]orthophosphate in DMEM phosphate-free medium for 12 h. Following [ $^{32}$ P]orthophosphate labeling, cells were pretreated for 2 h with MEM or MEM containing NDGA (10, 20, 50  $\mu$ M; **A**) or Trolox (100, 200, 500  $\mu$ M; **B**) and then subjected to co-treatment with vehicle or bleomycin (5  $\mu$ U) for 12 h in presence of 0.05% (v/v) 1-Butanol. At the end of incubation period, [ $^{32}$ P]PBt formed was determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Figure 6:** Bleomycin induces ROS generation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were preloaded with 10  $\mu$ M DCFDA for 30 min in complete MEM. Following the DCFDA loading, cells were subjected to treatment with vehicle or bleomycin (0-100  $\mu$ U) for 1 h (**A**) and 0.5 h (**B**). At the end of incubation period, the DCFDA fluorescence (as an index of ROS formation) was determined as described under Materials and Methods. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions. Data

represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone.

**Figure 7:** Bleomycin causes GSH depletion in ECs.

BLMVECs ( $1 \times 10^5$  cells/96 well plates) were subjected to treatment with vehicle or bleomycin (0-100  $\mu$ U) for 12 h. At the end of incubation period, the intracellular soluble thiol concentrations were determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone.

**Figure 8:** Bleomycin induces lipid peroxidation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were subjected to treatment with vehicle or bleomycin (0-100  $\mu$ U) for 12 h. At the end of incubation period, the cells were fixed, stained for 4HNE formation, and visualized using confocal microscopy as described under Materials and Methods. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

**Figure 9:** PLD inhibitor, FIPI, protects against bleomycin-induced PLD activation in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^{32}$ P]orthophosphate in DMEM phosphate-free medium along with pretreatment of MEM or MEM containing FIPI (250, 500, 1000 nM) for 12 h. Following the [ $^{32}$ P]orthophosphate labeling and FIPI pretreatment, cells were subjected to co-treatment with vehicle or bleomycin (5  $\mu$ U) for 12 h in presence of 0.05% (v/v) 1-Butanol. At the end of incubation period, [ $^{32}$ P]PBt

formed was determined as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Figure 10:** Bleomycin induces cytotoxicity and morphological alterations in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were subjected to treatment with vehicle or bleomycin (0-100  $\mu$ U) for 4-24 h. At the end of incubation period, the morphological pictures (as an index of cytotoxicity) were taken as described under Materials and Methods. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

**Figure 11:** PLD inhibitor protects against bleomycin-induced morphological alterations in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were pretreated with complete MEM or complete MEM containing FIPI (1  $\mu$ M) for 12 h and then subjected to co-treatment with vehicle or bleomycin (0-100  $\mu$ U) for 4-24 h. At the end of incubation period, the morphological pictures (as an index of cytotoxicity) were taken as described under Materials and Methods. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

**Figure 12:** Bleomycin induces cytotoxicity in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were subjected to treatment with MEM alone or MEM containing bleomycin (0-100  $\mu$ U) for 8 (A), 12 (B), and 24 (C) h. At the end of incubation period, release of LDH into the medium (as an index of cytotoxicity) was determined spectrophotometrically as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone.

**Figure 13:** PLD inhibitor, FIPI, protects against bleomycin-induced cytotoxicity in ECs.

BLMVECs ( $5 \times 10^5$  cells/35-mm dish) were pretreated with MEM alone or MEM containing FIPI (250, 500, 1000 nM) for 12 h. Following the pretreatment, cells were subjected to co-treatment with vehicle or bleomycin (5  $\mu$ U) for 12 h. At the end of incubation period, release of LDH into the medium (as an index of cytotoxicity) was determined spectrophotometrically as described under Materials and Methods. Data represent mean  $\pm$  S.D. calculated from three independent experiments. \*Significantly different at  $P < 0.05$  as compared to cells treated with vehicle alone. \*\* Significantly different at  $P < 0.05$  as compared to cells treated with bleomycin alone.

**Schema 1:** Proposed mechanism

Schematic representation of bleomycin-induced PLD activation and generation of lipid signal mediator, PA, and the subsequent lung endothelium damage.



## Hypothesis

Bleomycin

Reactive Oxygen Species  
(ROS)

Thiol-Redox Alterations

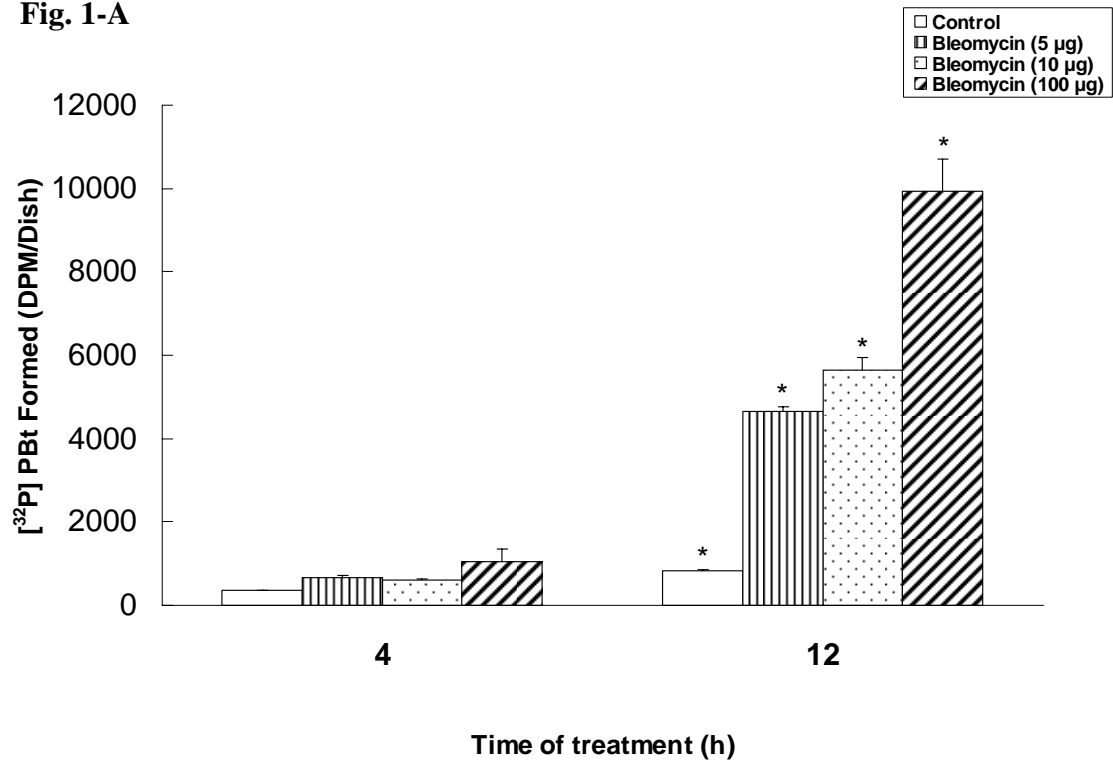
Phospholipase D activation

Lipid Signal mediator, PA

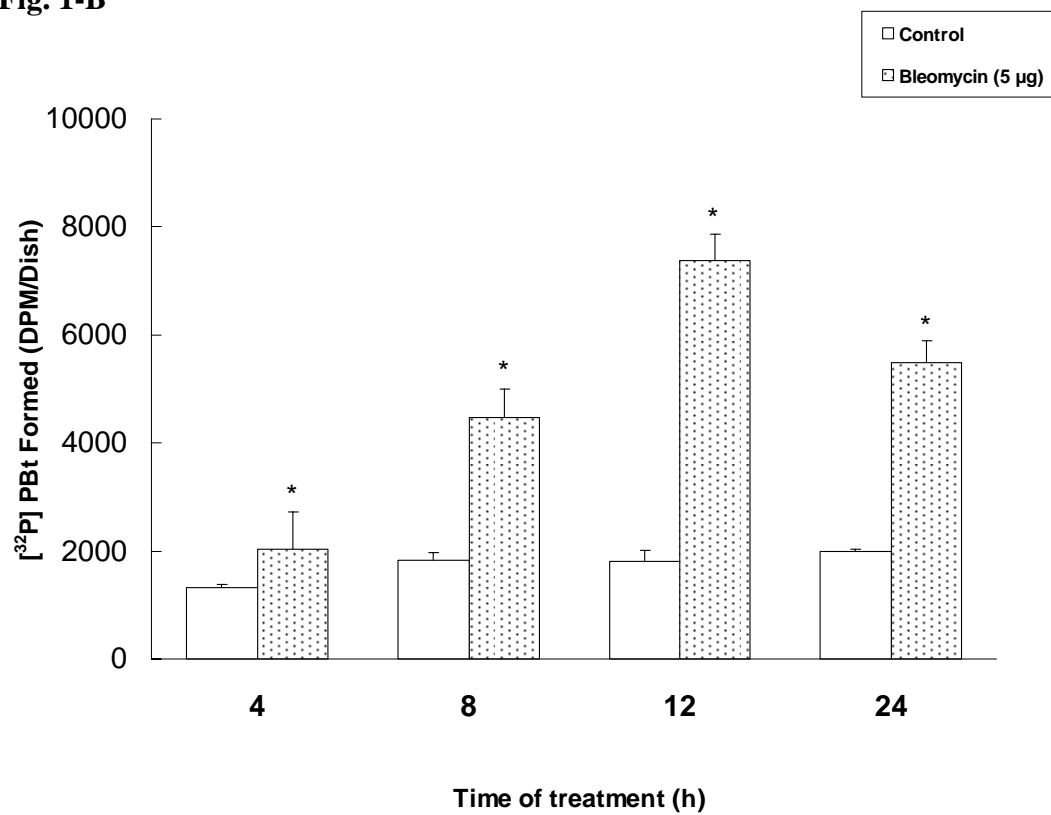
Endothelial Cell Damage

Blood Vessel Dysfunciton

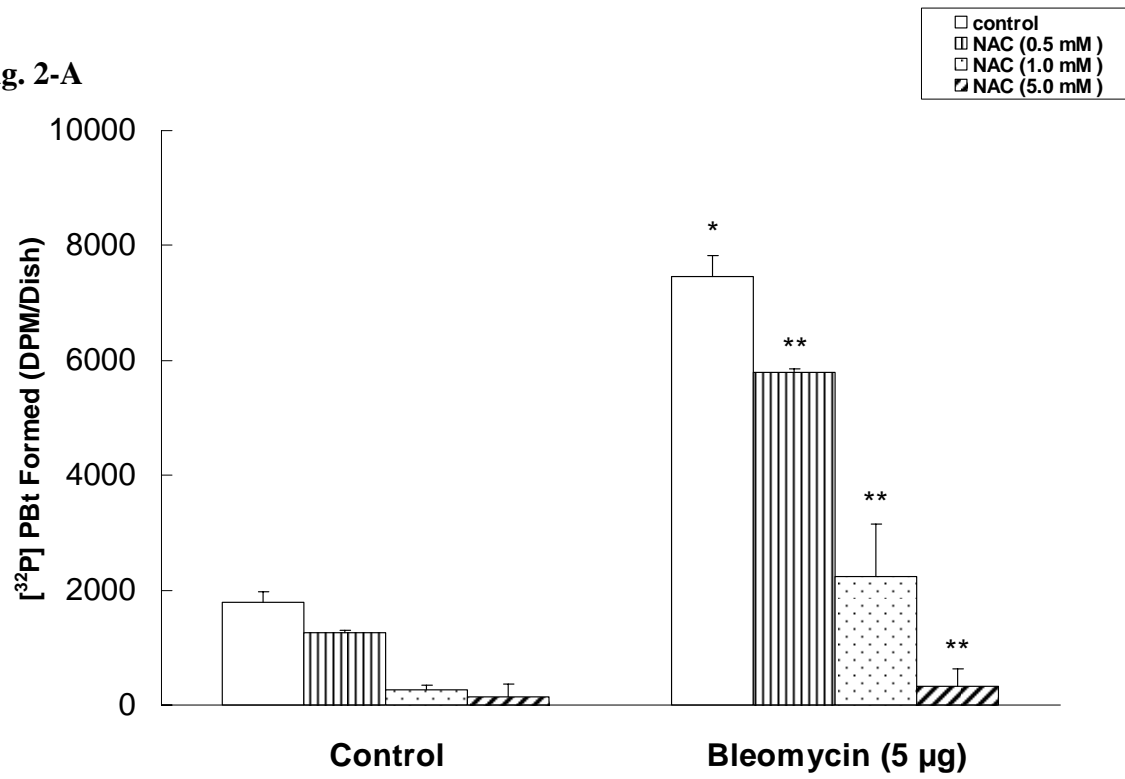
**Fig. 1-A**



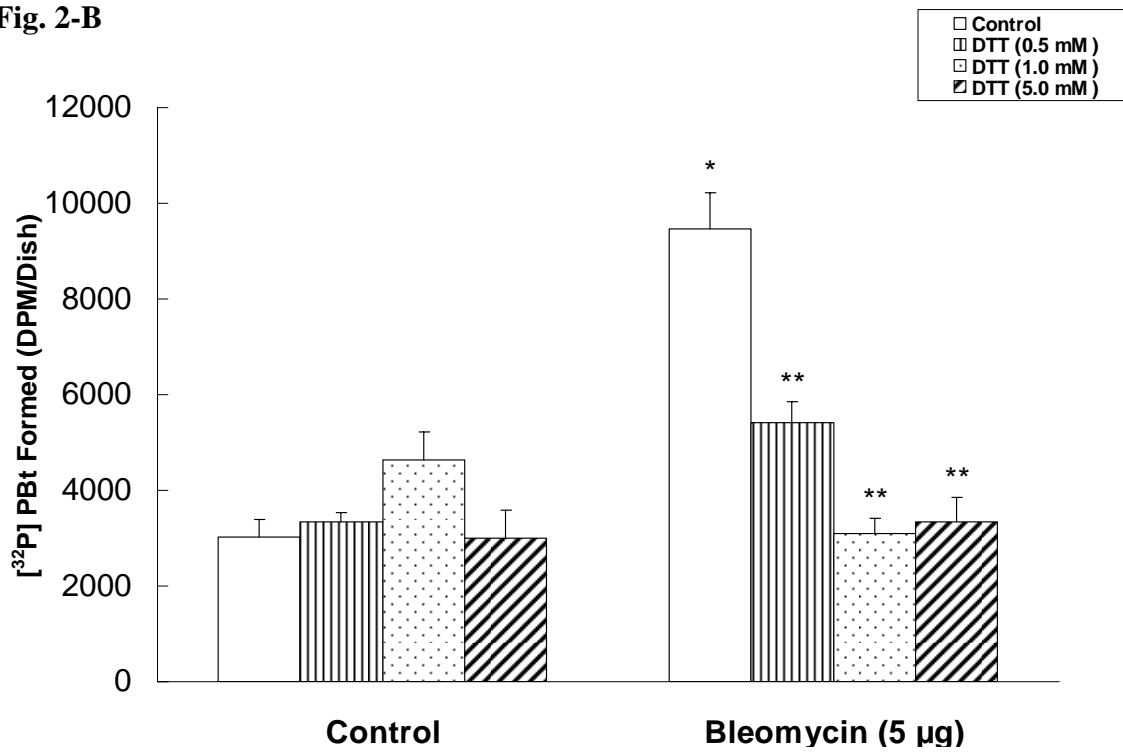
**Fig. 1-B**



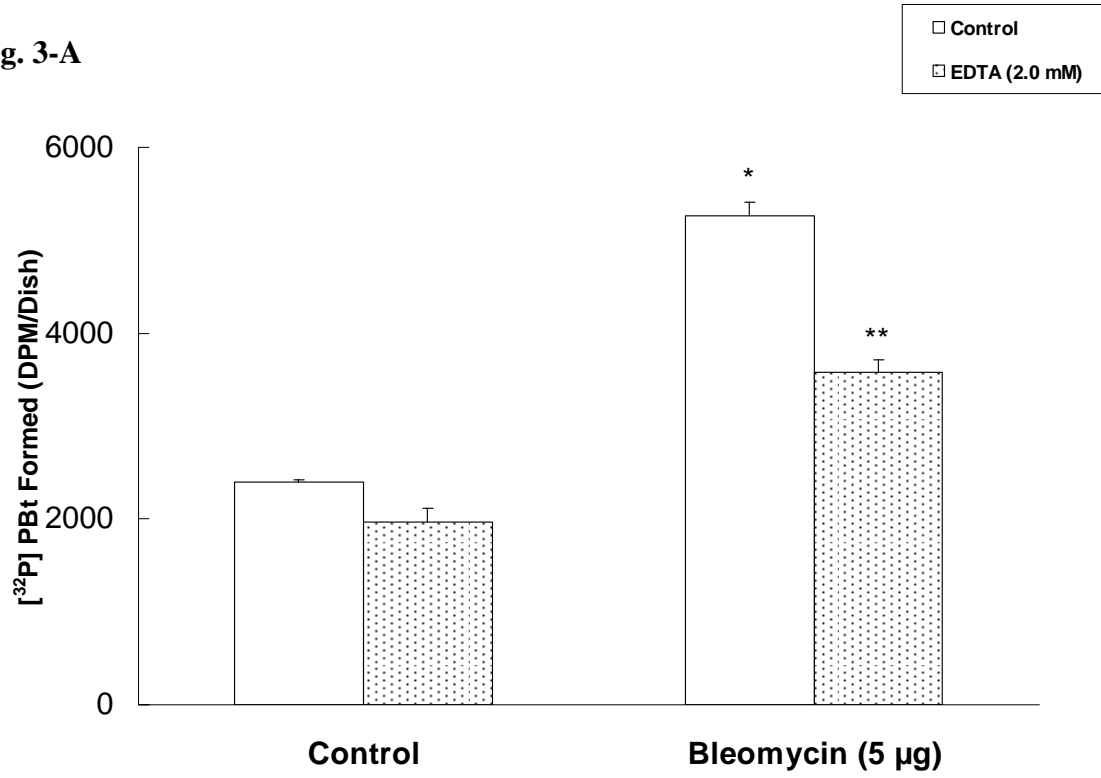
**Fig. 2-A**



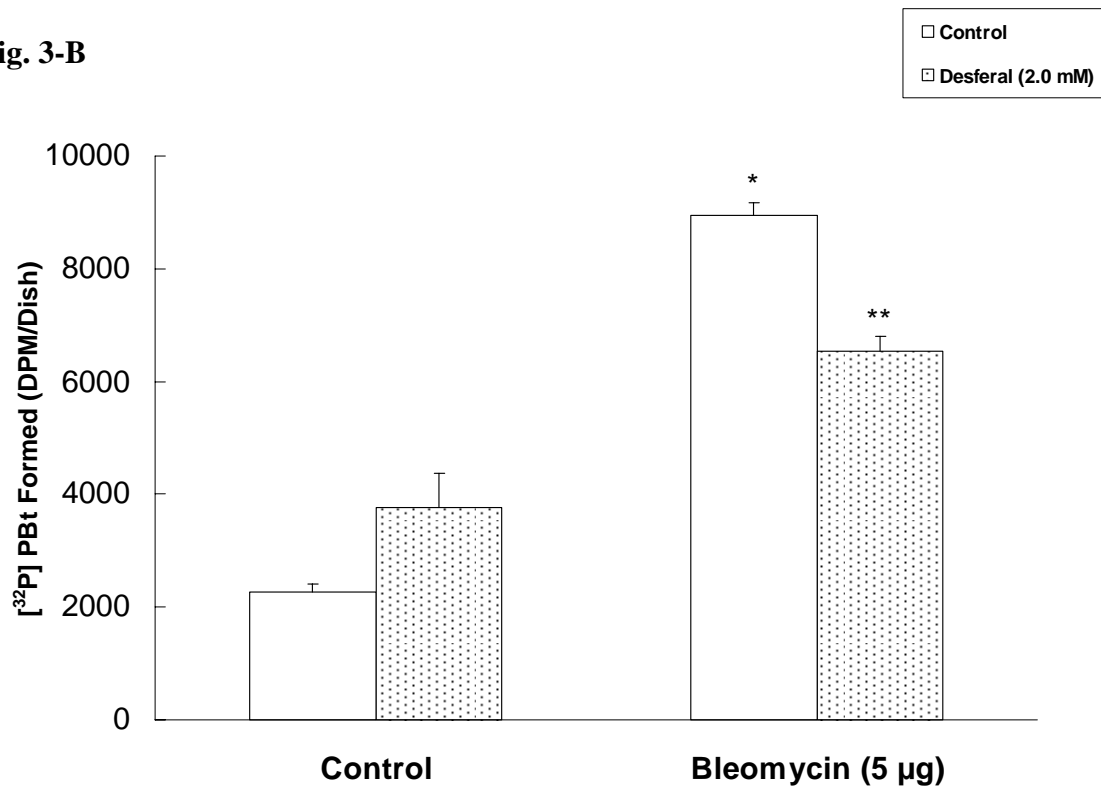
**Fig. 2-B**



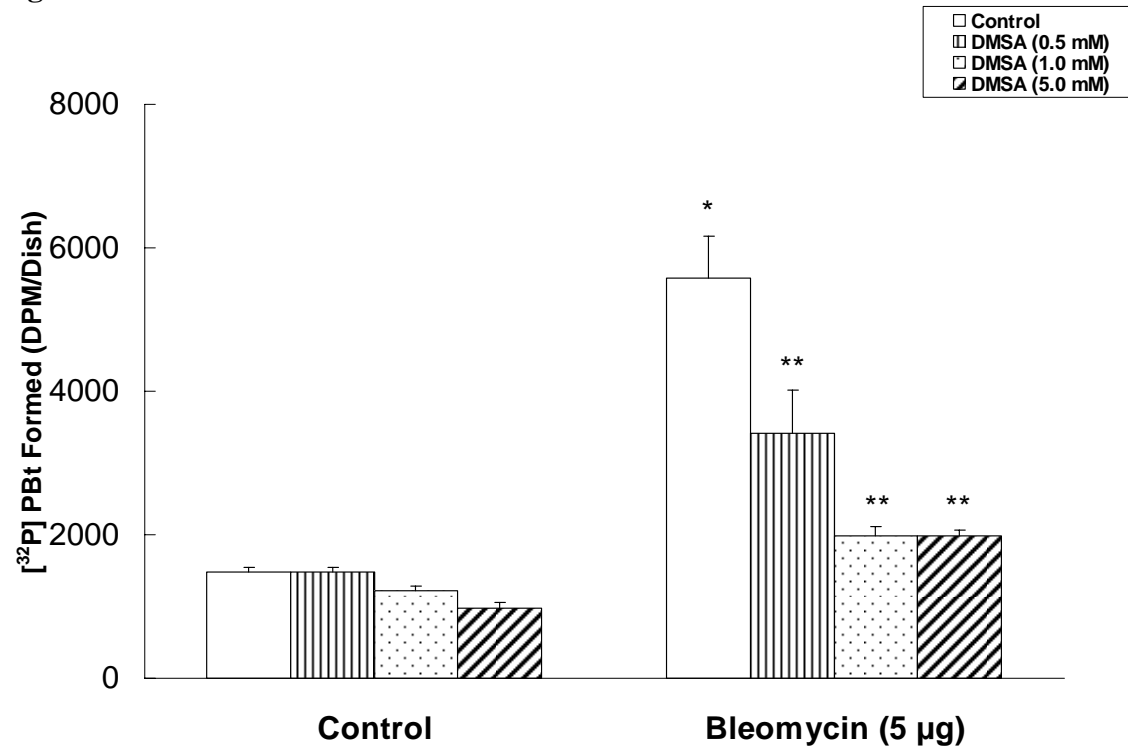
**Fig. 3-A**



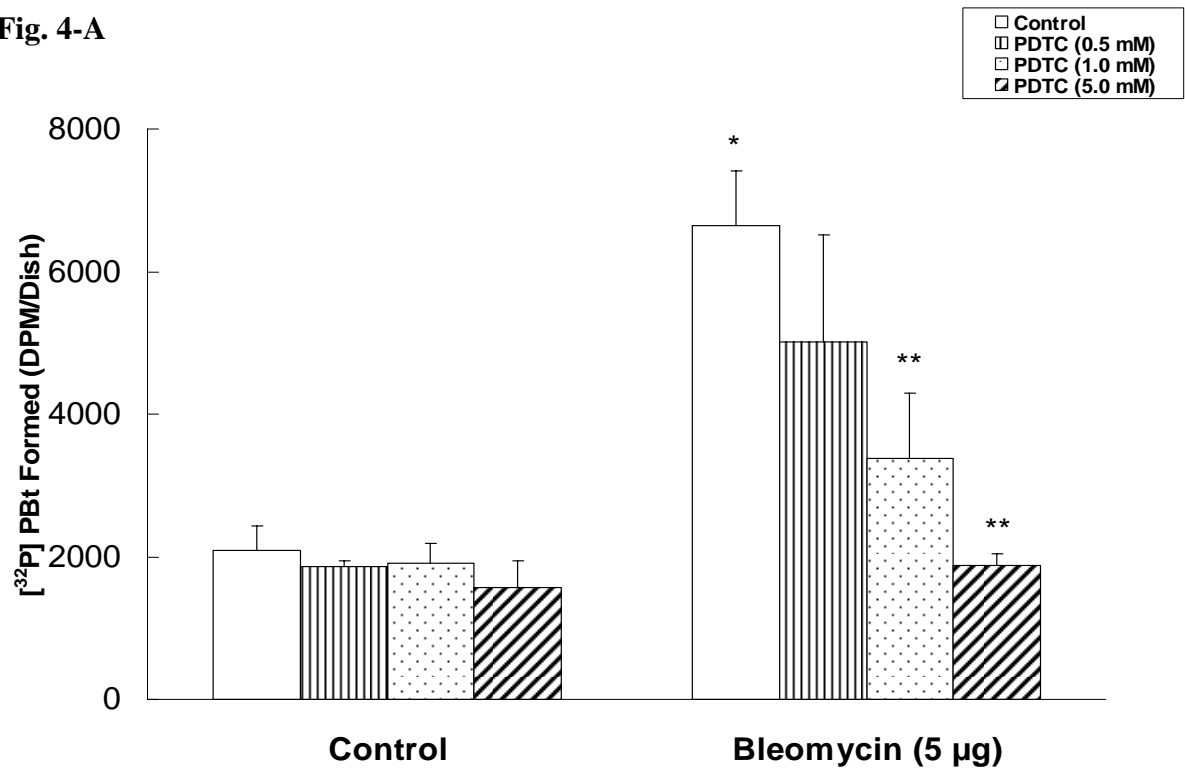
**Fig. 3-B**



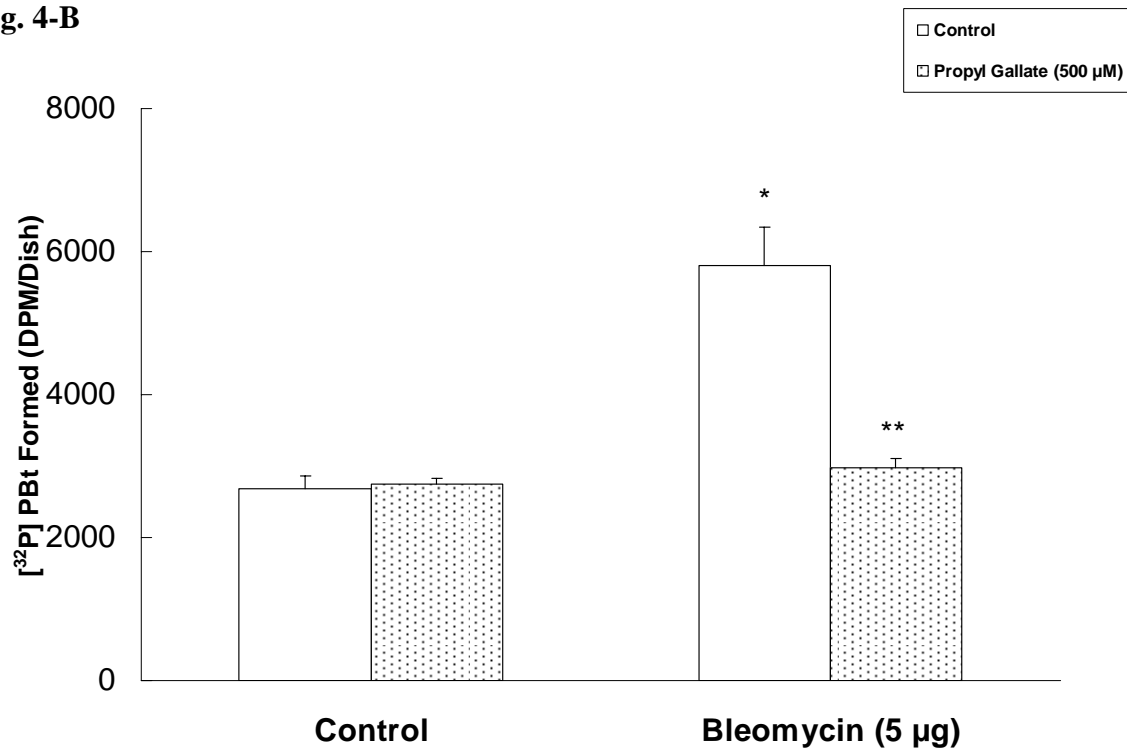
**Fig. 3-C**



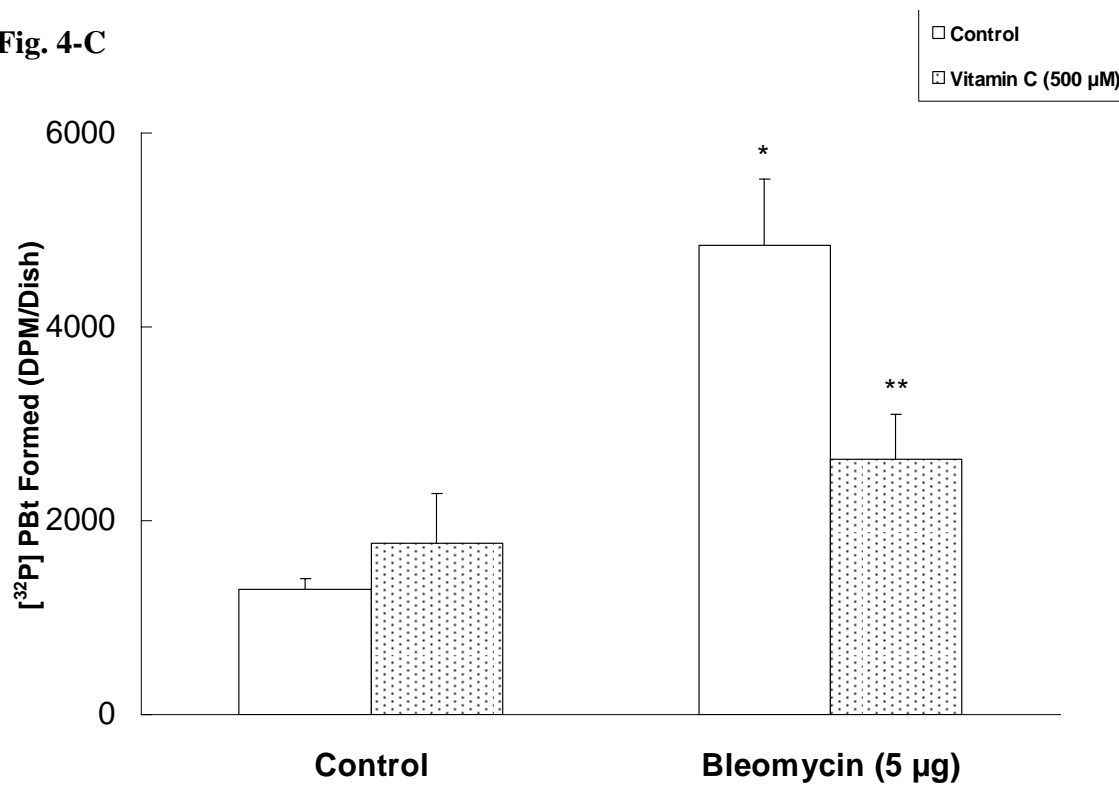
**Fig. 4-A**



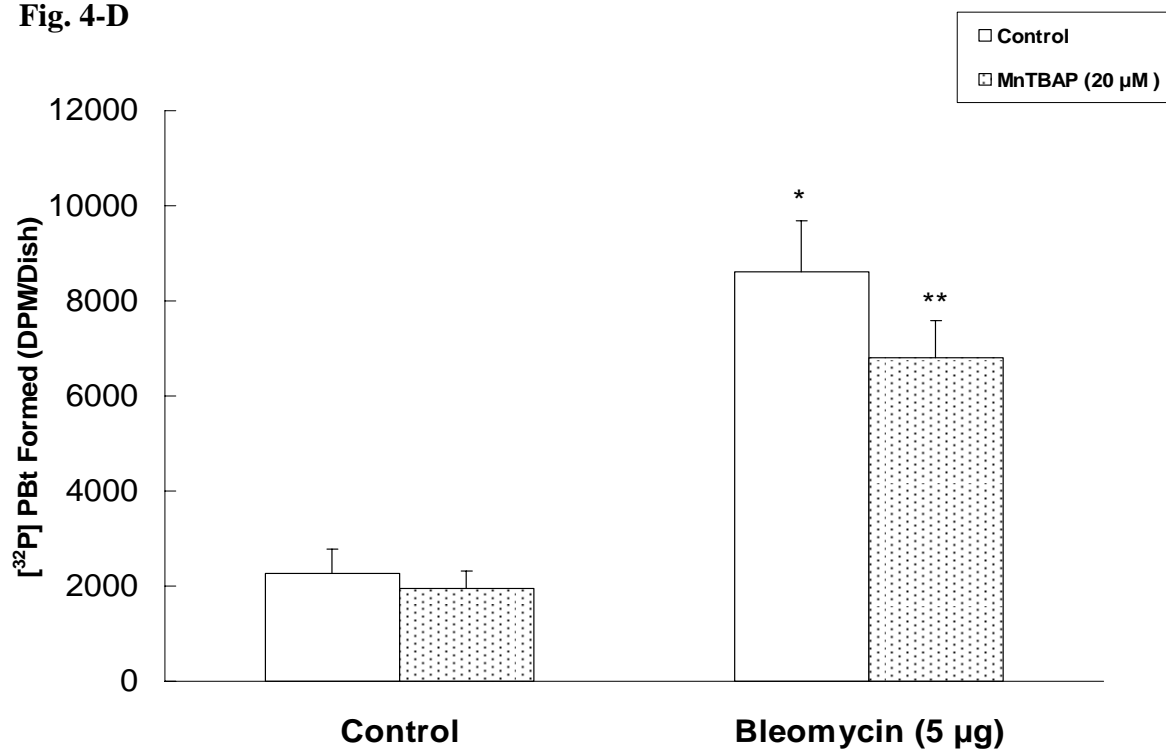
**Fig. 4-B**



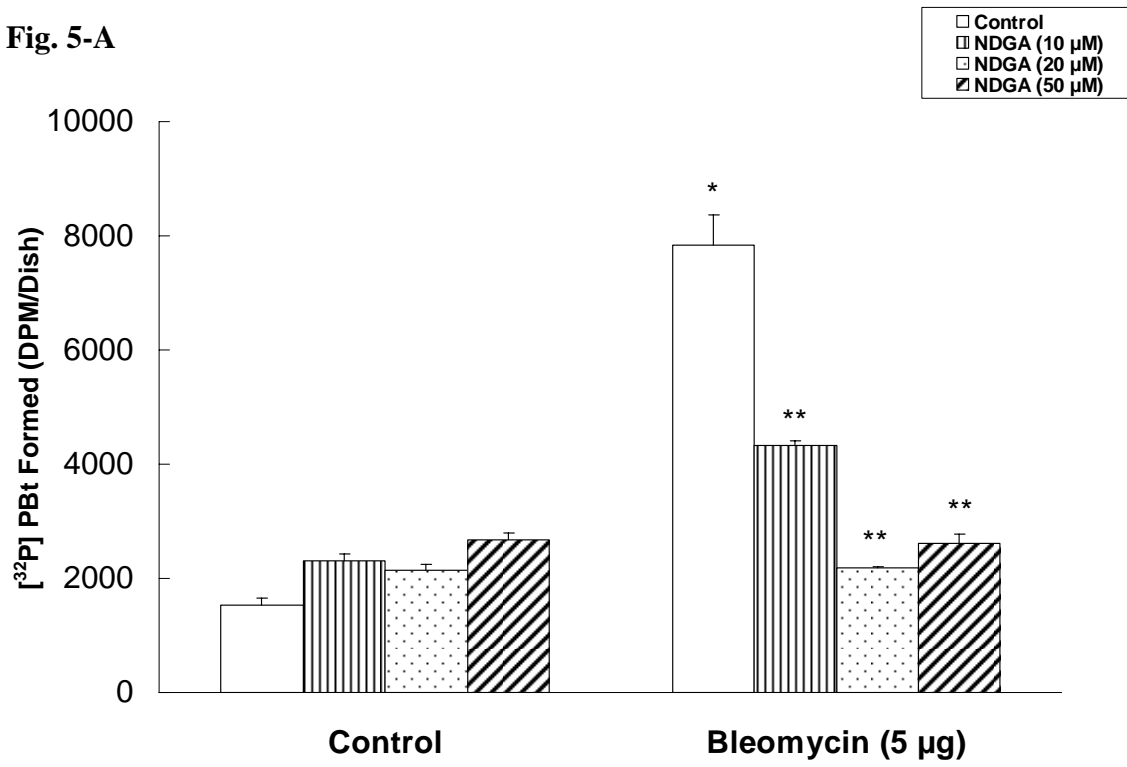
**Fig. 4-C**



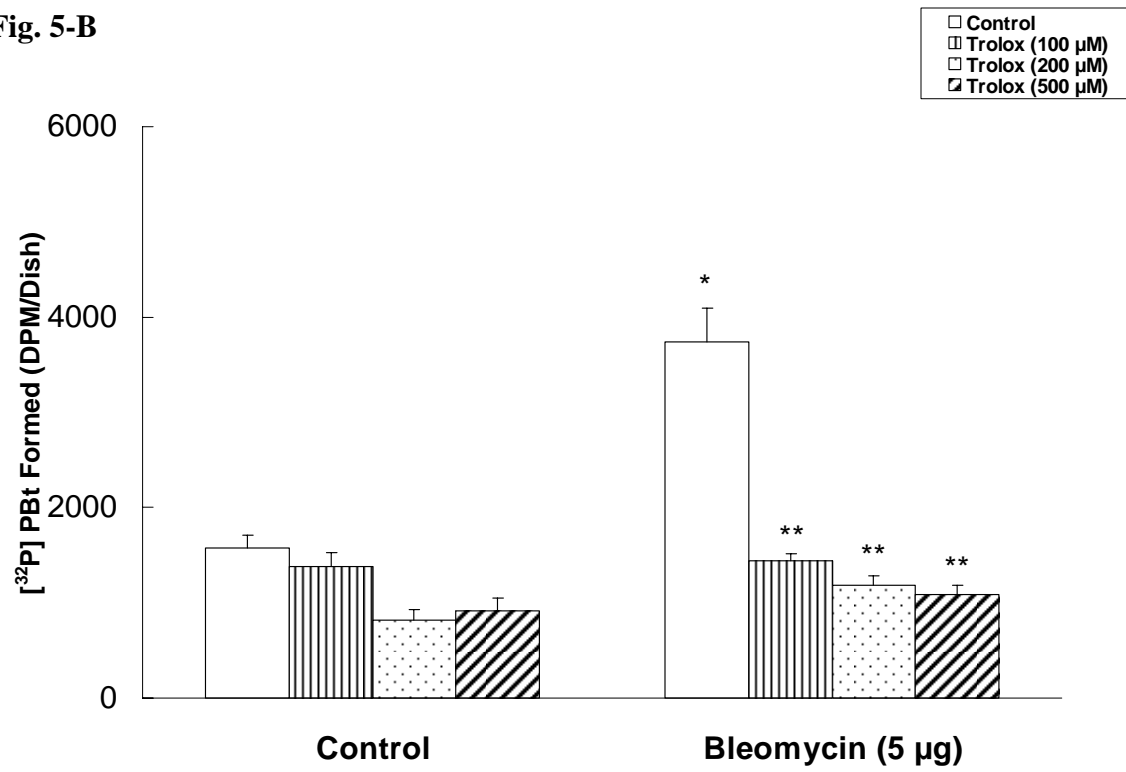
**Fig. 4-D**



**Fig. 5-A**

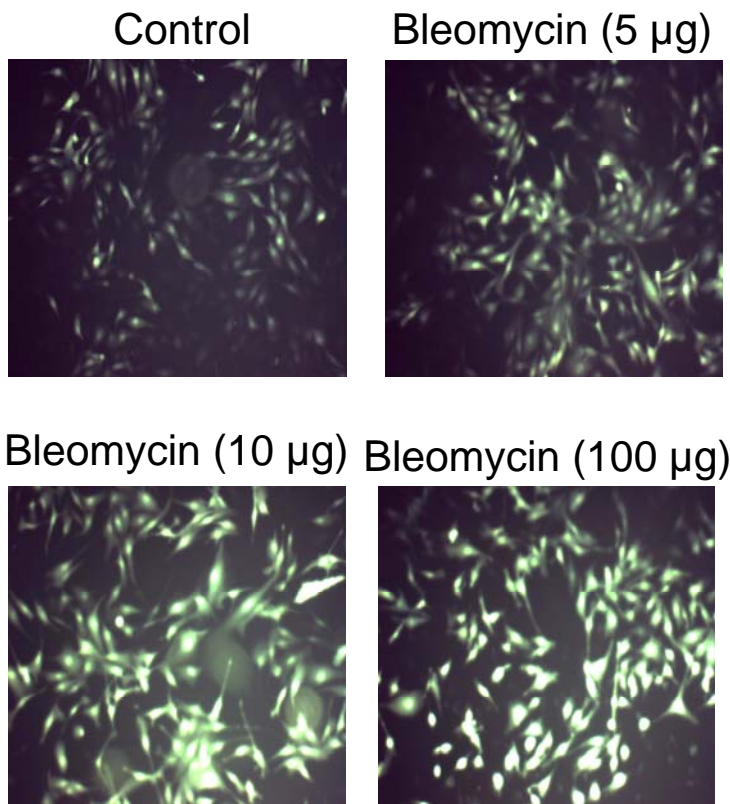


**Fig. 5-B**

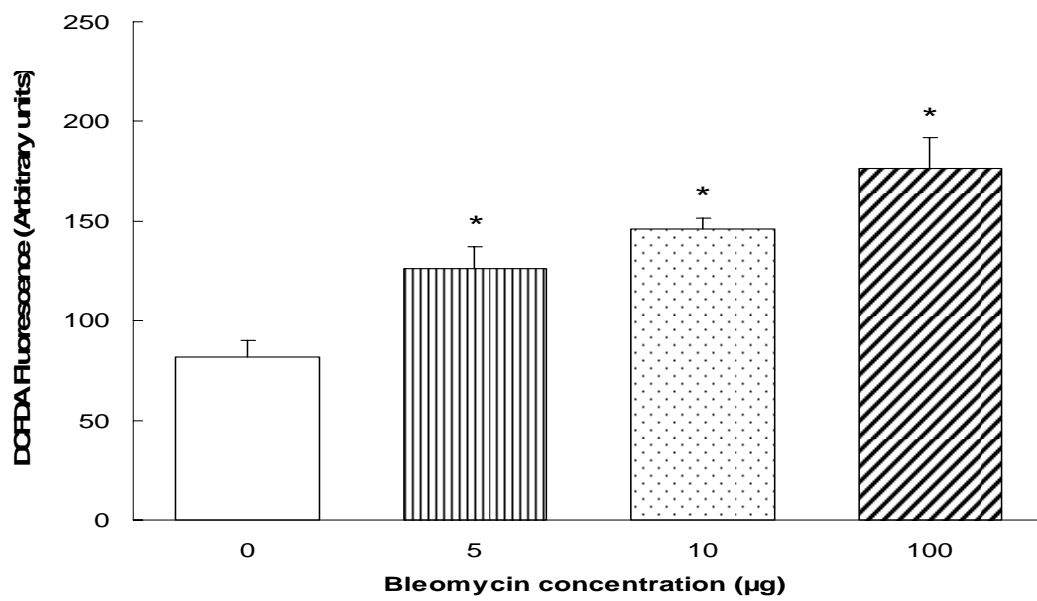




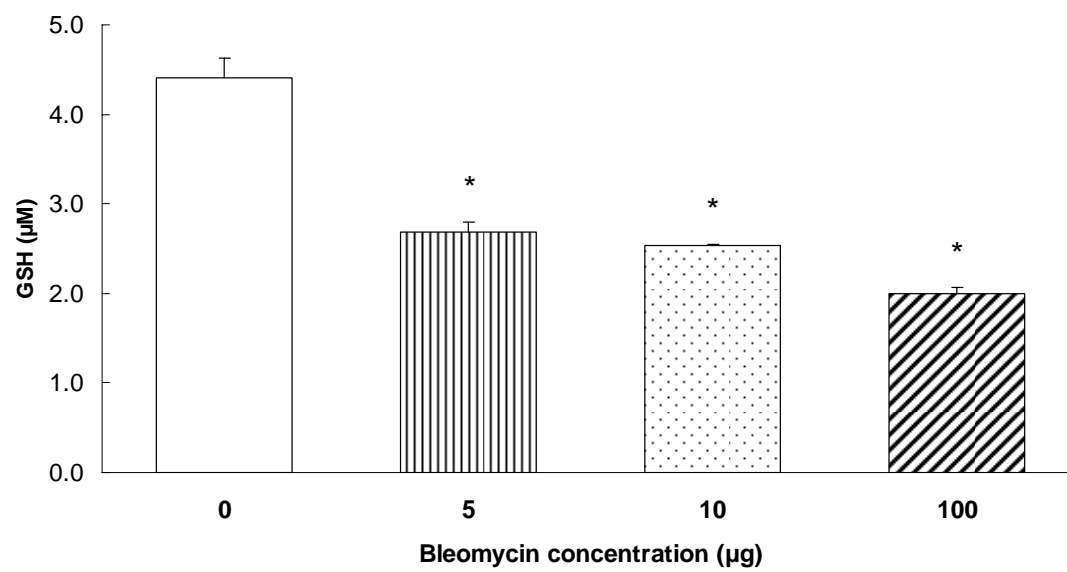
**Fig. 6-A**



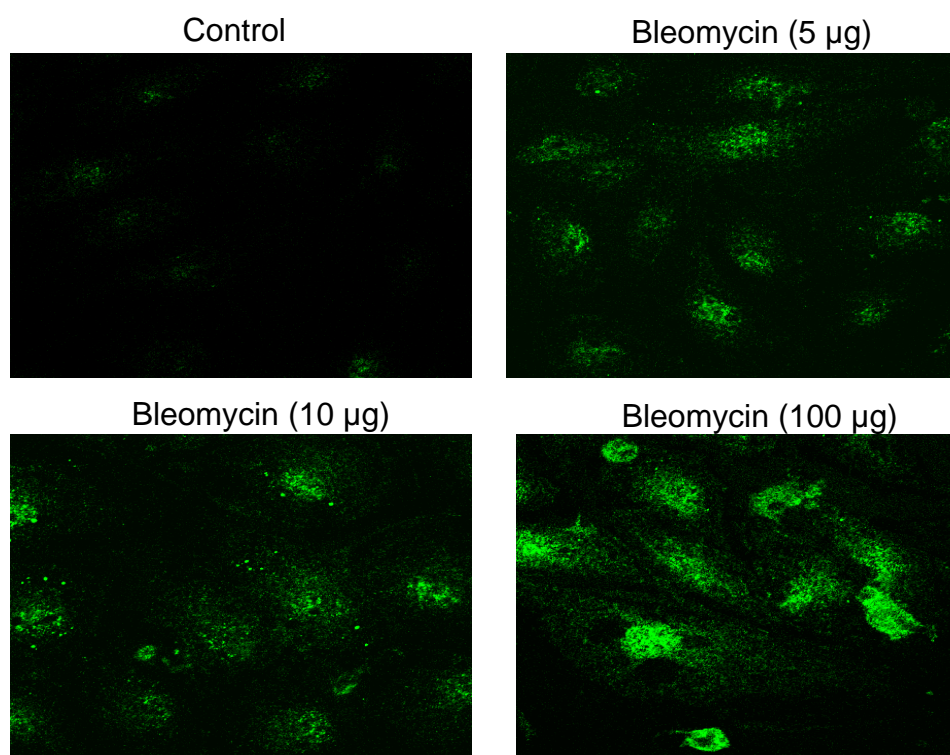
**Fig. 6-B**



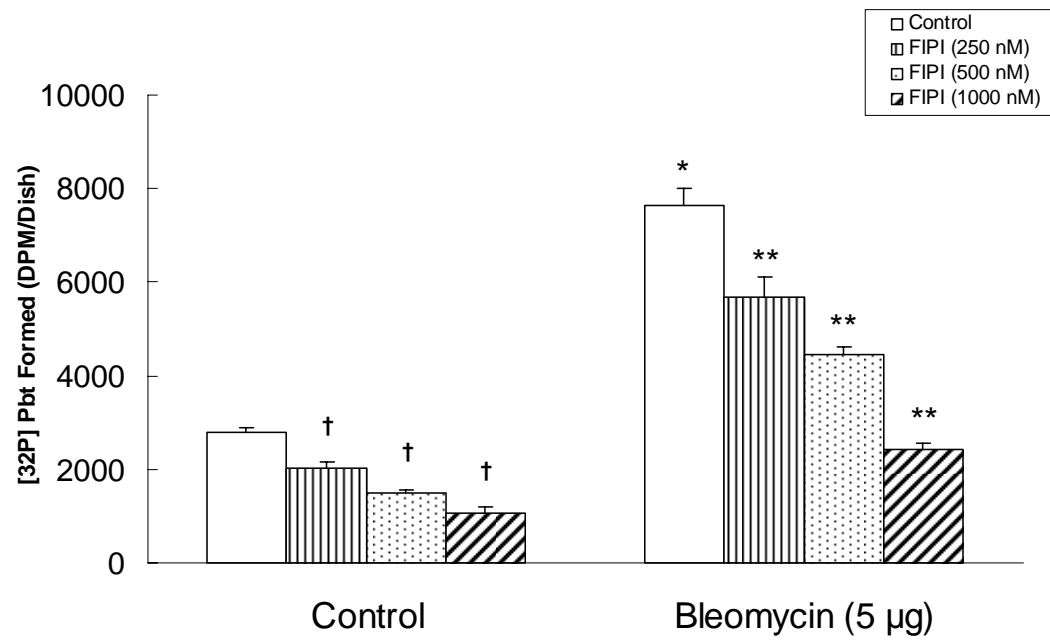
**Fig. 7**



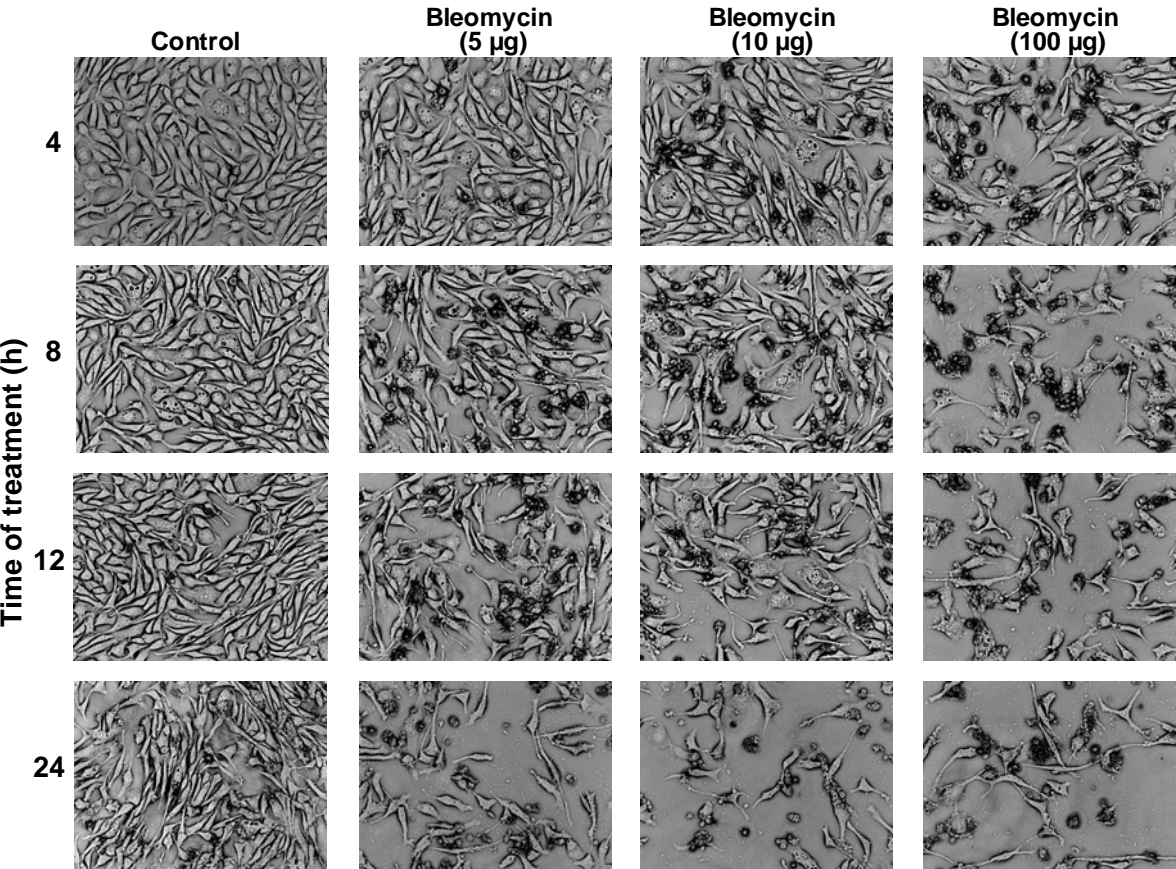
**Fig. 8**



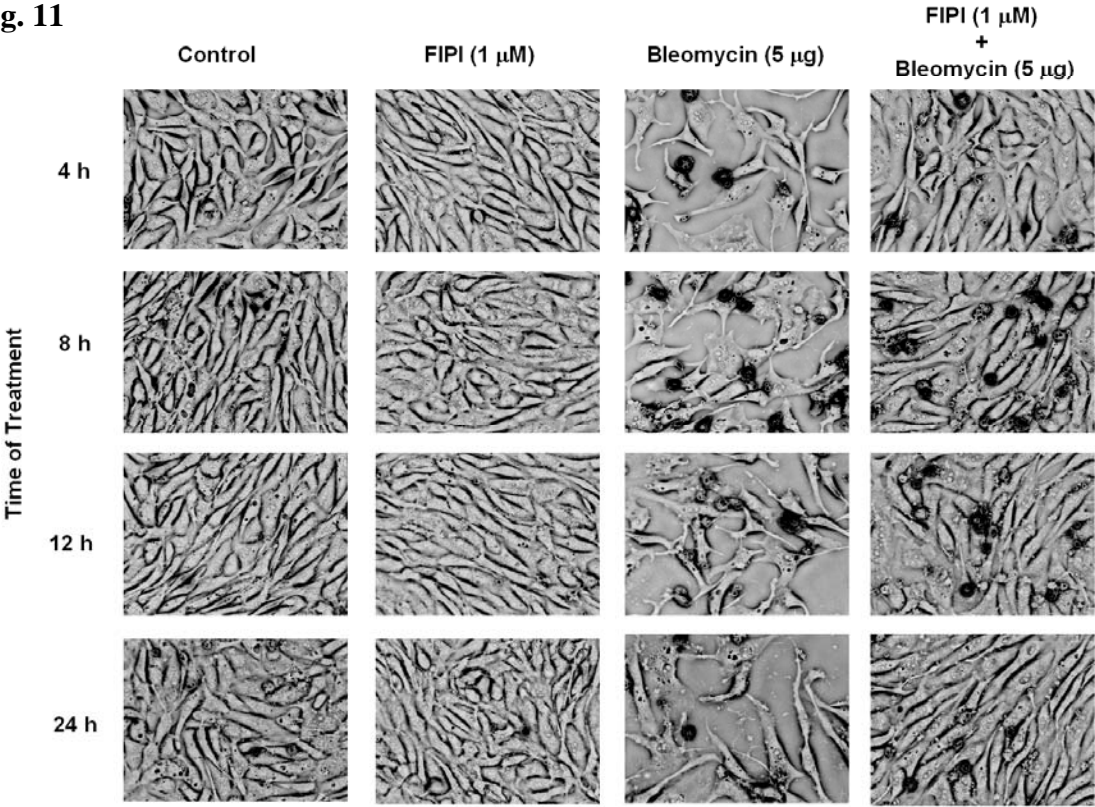
**Fig. 9**



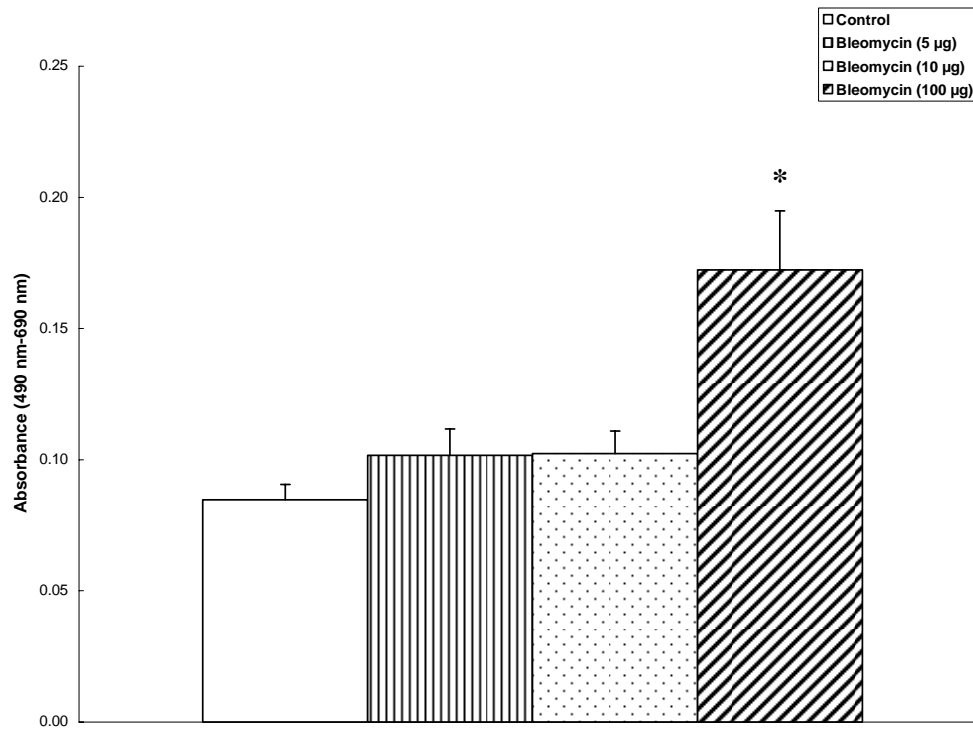
**Fig. 10**



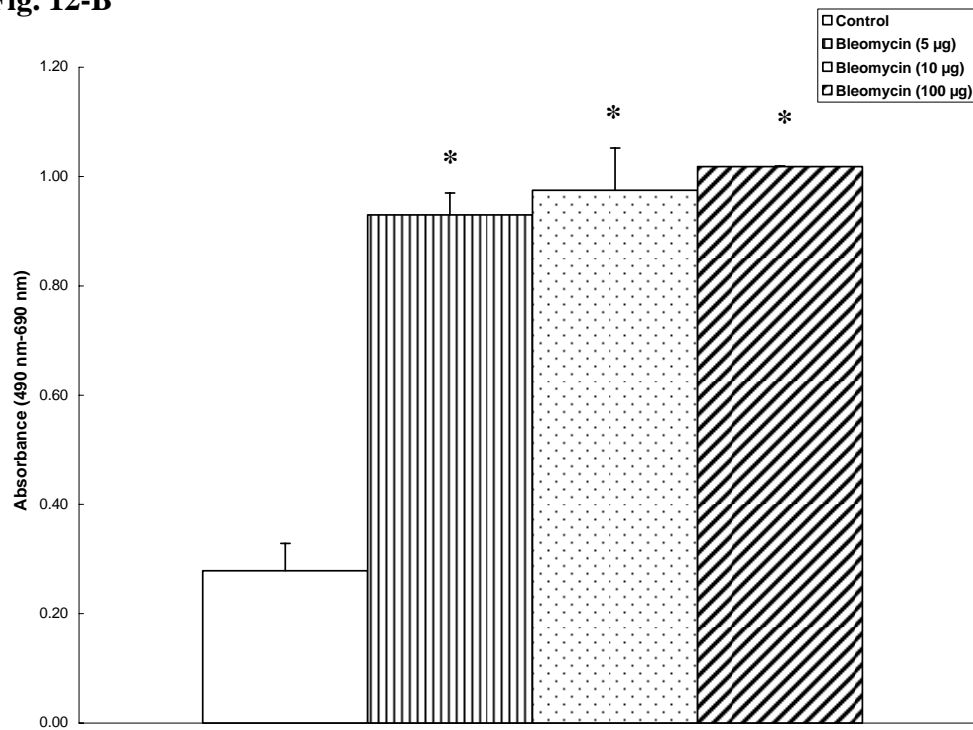
**Fig. 11**



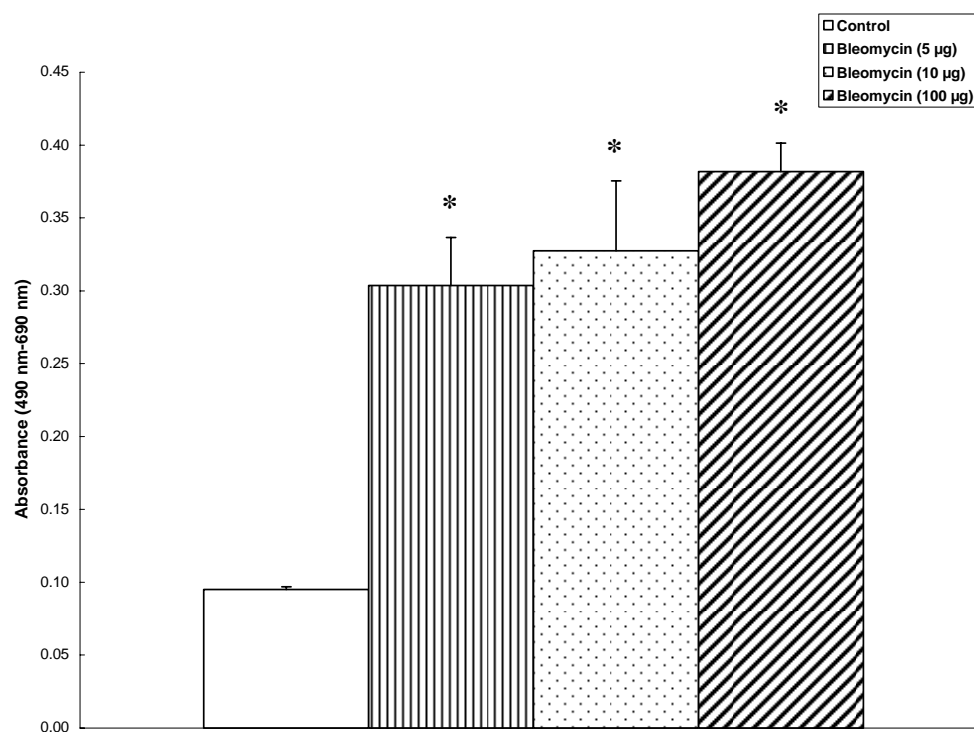
**Fig. 12-A**



**Fig. 12-B**

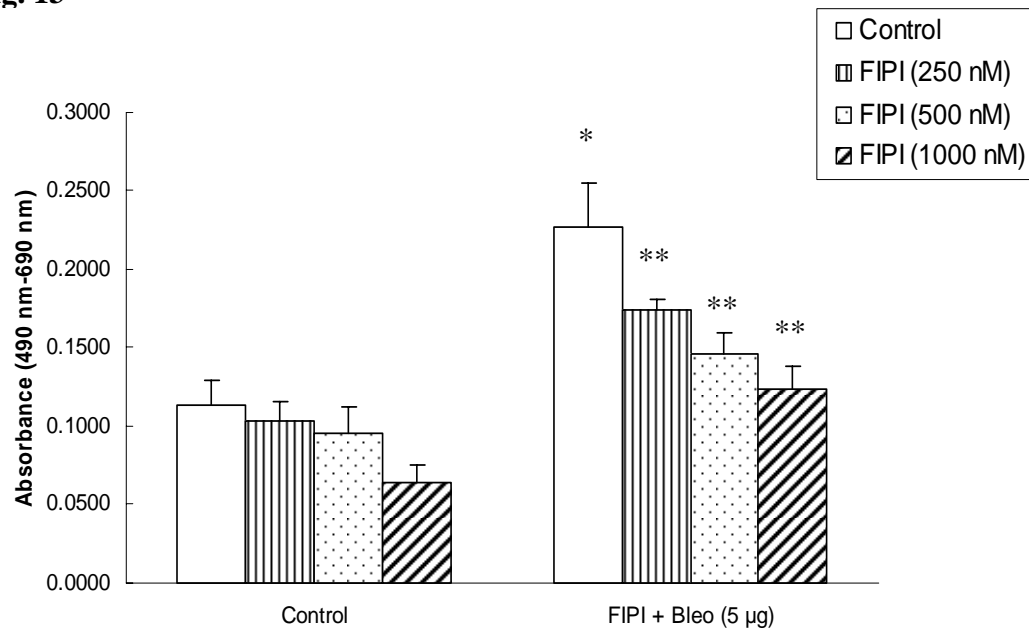


**Fig. 12-C**





**Fig. 13**



Schema 1

